METHOD FOR GENETIC ENGINEERING OF DISEASE RESISTANCE USING THE DRR206 CLASS OF PROTEINS

The present invention relates generally to the field of transgenic plants and expression vectors.

BACKGROUND OF THE INVENTION

Plant defense in response to pathogenic attack is accompanied by modification of plant cell structures, synthesis of secondary metabolites and accumulation of specific defense proteins (Staskawicz et al, 1995, *Science* **268**:661-667; Linthorst, 1991 *Crit Rev Plant Sci* **10**: 123-150). After infection by pathogens, inducible resistance often results in cell death at the infection site in order to restrict further pathogen growth. In some cases, the area of cell death develops a visible necrotic lesion of a hypersensitive response (HR) (Matthews, 1991, Plant Virology Academic Press: San Diego). During this response, genes encoding proteins related to defense are expressed (Ward et al, 1991, *Plant Cell* **3**:1085-1094). Since PR proteins with antimicrobial activity are induced during systemic acquired resistance and hypersensitive responses, these PR proteins may function in protecting plants from pathogenic attack (Lawton et al, 1993).

The simplest means for genetic engineering of resistance to fungal diseases entails the constitutive expression of one or more defense proteins in transgenic plants. Defense proteins with clearly demonstrated antifungal activities have been effective in several cases. For example, it has been shown that constitutive expression of bean chitinase protects transgenic tobacco seedlings from *Rhizoctonia solani* (Broglie et al, 1991, *Science* **254**:1194-1197). Similarly, transgenic tobacco plants expressing PR1a showed decreased or delayed symptoms both to blue mold (*Peronospora tabacina*) and black shank (*Phytophthora parasitica*) (Alexander et al, 1993, *Proc Natl Acad Sci USA* **90**:7327-7331). In potato, constitutive expression of osmotin delayed the onset of symptoms of potato late blight (*Phytophthora infestans*) (Liu et al, 1994, *Proc Natl Acad Sci USA* **91**:1888-1892). In addition, it has been demonstrated that constitutive expression of radish defensin protects transgenic tobacco from the foliar pathogen *Alternaria longipes* (Terras et al, 1995, *Plant Cell* **7**:573-588). While no direct antifungal activity has been demonstrated for PR10, constitutive expression of pea

PR10 in transgenic potato confers resistance to potato early drying disease (*Verticillium dahliae*) (Chang et al, 1993, *American Potato Journal* **70**:635-647). In a rare example in which an inducible gene was used, it was demonstrated that a genomic copy of the grape stilbene synthase gene confers resistance to *Botrytis cinerea* in transgenic tobacco (Hain et al, 1993, *Nature* **361**:153-156).

In some cases, genes other than defense genes have been useful in plant protection. Constitutive expression of the fungal elicitor protein β-crytogein from *Phytophthora cryptogea* conferred resistance to *Phytophthora parasitica* in transgenic tobacco. In potato, constitutive expression of the fungal glucose oxidase gene, which generates H_2O_2 from glucose, resulted in resistance to both *Phytophthora infestans*, as well as to the bacterium *Erwina carotovora* (Wu et al, 1995, *Plant Cell* **7**:1357-1368).

US Patent 5,312,912 issued to Hadwiger et al. describes the DNA sequence of the pea genomic clone DRR206 and suggests methods for transferring to plants the unique DNA sequences DRRG49 and DRR206. While Hadwiger does speculate that transformation of potato or tobacco with DRRG49 or DRR206 genes or other proteins driven by either the DRRG49 or DRR206 promoters might provide resistance to infection, no proof is given that this is indeed the case. That is, while a proposed method is described, no examples of this method being used successfully are provided. Specifically, Hadwiger deals primarily with methods for improving expression of the DRRG49 promoter by incorporating new elements into the control sequence.

Wang and Fristensky on July 7, 1997 (Canadian Phytopathological Society Annual Meeting, Winnipeg, Canada) reported preliminary data suggesting a lower rate of spore germination of PG2 pycnidiospores of *L. maculans* in extracts from a single *Brassica napus* line transformed with 35S-DRR206 and a single *Brassica napus* line transformed with 35S-defensin. However, all experiments were done with parental lines and no evidence was provided indicating that the defensin and DRR206 proteins were being expressed.

While such results are encouraging, there is no guarantee that a protein that was effective in one host against one pathogen will be effective in a different host against a different pathogen. For example, the same osmotin

construct that conferred resistance to *P. infestans* in transgenic potato had no effect on *P. parasitica* in transgenic tobacco (Liu et al, 1994). Similarly, constitutively-expressed potato PR10 had no effect on resistance to the late blight pathogen *Phytophthora infestans* (Constabel et al, 1993, *Plant Mol Bio* **22**:775-782). We have therefore tested four genes from pea for their effectiveness against the blackleg fungus, *Leptosphaeria maculans*, in transgenic canola (*Brassica napus*). Three of the genes, PR10.1, chitinase and defensin, have previously demonstrated effectiveness in other plant/pathogen systems, as discussed above. The fourth gene, DRR206 has not previously been used in transgenic plant experiments, although the gene is strongly induced in pea in response to both fungal and bacterial pathogens and elicitors (Riggleman et al, 1985, *Plant Mol Bio* **4**:81-86; Fristensky et al, 1985, *Physiological Plant Pathology* **27**:15-28; Daniels et al, 1987, *Plant Mol Biol* **8**:309-316; Hadwiger et al, 1992, *Physiol Mol Plant Pathol* **40**:259-269; Cody et al, 1988, *Phytopathology* **78**:1451-1453). We now demonstrate that constitutive expression of pea DRR206 and defensin each can confer substantial resistance to *L. maculans* in transgenic *B. napus*.

SUMMARY OF THE INVENTION

According to an aspect of the invention, there is provided a recombinant expression system, capable, when transformed into a plant, of expressing a DNA sequence encoding DRR206 protein, said DRR206 protein having at least 60% identity to amino acids 1-184 of Figure 8, which system comprises control sequences effective in said plant operably linked to said DNA sequence.

The plant may be *Brassica napus*.

The control sequences may include a strong constitutive promoter, for example the 35S promoter.

The control sequences may include an inducible promoter.

The inducible promoter may be responsive to pathogen infection.

The expression system may include T-DNA for integration of the expression system into a plant genome.

According to a second aspect of the invention, there are provided

transgenic plants and plant cells containing the above-described expression system. The plants or plant cells may be *B. napus*..

According to a third aspect of the invention, there is provided a recombinant expression system, capable, when transformed into a plant, of expressing a DNA sequence encoding defensin protein, said defensin protein having at least 60% identity to amino acids 1-72 of Figure 9, which system comprises control sequences effective in said plant operably linked to said DNA sequence.

The plant may be *Brassica napus*.

The control sequences may include a strong constitutive promoter, for example, the strong constitutive promoter is the 35S promoter.

The control sequences may include an inducible promoter. The inducible promoter may be responsive to pathogen infection.

The recombinant expression system may include T-DNA for integration of the expression system into a plant genome.

According to a fourth aspect of the invention, there are provided transgenic plants and plant cells containing the above-described expression system. The plants or plant cells may be *B. napus*.

According to a fifth aspect of the invention, there is provided a method for producing a plant with improved disease resistance comprising transforming a plant with an expression system comprising a DNA sequence encoding DRR206 protein operably linked to control sequences effective in said plant, said DRR206 protein having at least 60% identity to amino acids 1 to 184 of Figure 8; and growing the plant under conditions such that the DRR206 protein is expressed.

The plant may be resistant to a pathogenic organism selected from the group consisting of *Rhizoctonia solani*, *Leptosphaeria maculans* isolate PG3, *Leptosphaeria mculans* isolate PG4 and *Sclerotinia sclerotiorum*.

According to a sixth aspect of the invention, there are provided plants and seeds produced according to the above-described method.

According to a seventh aspect of the invention, there is provided a method for producing a plant with improved disease resistance comprising

transforming a plant with an expression system comprising a DNA sequence encoding defensin protein operably linked to control sequences effective in said plant, said defensin protein having at least 60% identity to amino acids 1 to 72 of Figure 9; and growing the plant under conditions such that the DRR206 protein is expressed.

The plant may be resistant to a pathogenic organism selected from the group consisting of *Rhizoctonia solani*, *Leptosphaeria maculans* isolate PG3, *Leptosphaeria mculans* isolate PG4 and *Sclerotinia sclerotiorum*.

According to an eighth aspect of the invention, there are provided plants and seeds produced according to the above-described method.

According to a ninth aspect of the invention, there is provided a method for producing a transgenic *B. napus* plant comprising: providing seeds of a *B. napus* strain; providing a recombinant expression system, capable, when transformed into *B. napus*, of expressing a DNA sequence encoding DRR206 protein, said DRR206 protein having at least 60% identity to amino acids 1 to 184 of Figure 8, which system comprises control sequences effective in *B. napus* operably linked to said DNA sequence; transfecting *Agrobacterium* with the recombinant expression system; germinating the *B. napus* seeds; removing cotyledons from the germinated seeds; cocultivating the cotyledons with the transfected *Agrobacterium*; regenerating shoots and roots from the cotyledons; and growing the transgenic *B. napus* plants to maturity.

According to a tenth aspect of the invention, there is provided a method of providing resistance in *B. napus* to a pathogenic organism comprising: providing seeds of a *B. napus* strain; providing a recombinant expression system, capable, when transformed into *B. napus*, of expressing a DNA sequence encoding DRR206 protein, said DRR206 protein having at least 60% identity to amino acids 1-184 of Figure 8, which system comprises control sequences effective in *B. napus* operably linked to said DNA sequence; transfecting *Agrobacterium* with the recombinant expression system; germinating the *B. napus* seeds; removing cotyledons from the germinated seeds; cocultivating the cotyledons with the transfected *Agrobacterium*; regenerating shoots and roots from the cotyledons; growing the transgenic *B. napus* plants such that DRR206 is

expressed within the cells of the transgenic *B. napus* plant, thereby preventing infection of the transgenic *B. napus* plant by the pathogenic organism.

The pathogenic organism may be selected from the group consisting of *Rhizoctonia solani*, *Leptosphaeria maculans* isolate PG3, *Leptosphaeria mculans* isolate PG4 and *Sclerotinia sclerotiorum*.

According to an eleventh aspect of the invention, there is provided a method of reducing damage to a plant by a pathogenic organism comprising: providing a plant having cells arranged to contain elevated levels of DRR206 protein, said DRR206 protein having at least 60% identity with amino acids 1-184 of Figure 8; and growing the plant such that DRR206 is expressed within the cells of the plant, thereby preventing or inhibiting growth of the pathogenic organism.

The pathogenic organism may be selected from the group consisting of *Rhizoctonia solani*, *Leptosphaeria maculans* isolate PG3, *Leptosphaeria maculans* isolate PG4 and *Sclerotinia sclerotiorum*.

One embodiment of the invention will now be described in conjunction with the accompanying drawings in which:

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram of the constitutive defense gene constructs in pBI121.

Figure 2 is a bar graph summarizing the distribution of copy number in transgenic lines.

Figure 3 is a bar graph of disease score and mRNA expression in transformed lines.

Figure 4 summarizes infection phenotypes of transgenic and untransformed *B. napus* inoculated with *Leptosphaeria maculans* PG2 (Rimmer and van den Berg, 1992, *Can J Plant Pathol* **14**:56-66). Specifically, Figure 4a-c shows inoculation of cotyledons by pinprick, wherein 4a is untransformed Westar, 4b is defensin transformant GN4-2#15 and 4c is DRR206 transformant GN3-4#22; Figure 4d shows adult plants inoculated at stems (left to right: untransformed Westar, GN4-2#15 with defensin, GN3-2#22 with DRR206; Figure 4e-g shows close-ups of stems shown in Figure 4d; Figure 4h-l shows hyphal growth in

cotyledon assay 8 days postinoculation; Figure 4k shows inoculation of cotyledons by infiltration 8 days postinoculation.

Figure 5 is an immunoblot of DRR206 proteins in transgenic *B. napus* and *Fusarium*-inoculated pea.

Figure 6 is a bar graph showing disease score of DRR206-transformed or untransformed *B. napus* inoculated with *L. maculans* PG3.

Figure 7 is a bar graph showing disease score of DRR206-transformed or untransformed *B. napus* inoculated with *Sclerotinia sclerotiorum*.

Figure 8 shows the protein and DNA sequences of DRR206.

Figure 9 shows the protein and DNA sequences of defensin.

Table 1 summarizes specific RNA accumulation and disease scores in *B. napus* transformed with defense genes.

Table 2 shows that disease score co-segregates with defense T-DNA insertions in T2 plants.

Table 3 shows germination rate of pycnidiospores of *L. maculans* and dry weight of hyphae of *L. maculans*.

Table 4 shows disease scores of adult transgenic plant stems inoculated with PG3.

Table 5 shows disease scores of transgenic plants inoculated with PG4.

Table 6 shows death rates of transgenic plants inoculated with *Rhizoctonia solani*.

Table 7 shows lesion sizes of transgenic plants inoculated with *Sclerotinina sclerotiorum*.

DETAILED DESCRIPTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference.

DEFINITIONS

As used herein, the term "canola" refers to specific cultivars of *Brassica napus* and *Brassica rapa* having low erusic acid and low liolenic acid..

As used herein, the term "preventing infection" refers generally to providing resistance to disease caused by pathogens, where disease resistance consists of a reduction of symptoms caused by infection, prevention of sporulation or a reduction in damage to the plant by the pathogen. These symptoms may include but are not limited to necrosis, tissue collapse, growth of the pathogen within plant tissues, and decreases in yield and/or quality due to infection.

As used herein, "mode of pathogenicity" refers to the part of a plant infected by a pathogen.

Expression vectors comprising protein coding sequences for PR10, chitinase, DRR206 and defensin were cloned into a T-DNA-based binary vector under control of a strong constitutive promoter, in this case, the 35S promoter. The vectors were then transformed into *B. napus* under conditions wherein the T-DNA regions integrated into the *B. napus* genome. Transformed plants were screened for cell lines containing single copies of the genes. Cell lines were then assayed for resistance to *Leptosphaeria maculans*, the blakleg fungus. Results indicate that lines transformed with PR10 or chitinase showed little or no inhibition of infection while lines transformed with DRR206 or defensin did. Inhibition of fungal growth or germination by extracts from defensin transgenic plants is less pronounced than that seen with DRR206 and appears to be qualitatively different therefrom as described below. Thus, *B. napus* plants expressing DRR206 are resistant to *L. maculans* infection. Furthermore, transgenic *B. napus* plants were also shown to be resistant to another *L. maculans* isolate, PG3, and to the fungi *Sclerotinia sclerotiorum* and *Rhizoctonia solani*, indicating that DRR206 may confer resistance to other pathogenic organisms as well.

While naturally-occurring genes for resistance to blackleg exist in some cultivars of *B. napus*, breeding these genes into other cultivars is a difficult process requiring years and many generations of crossing. Specifically, most of the work is to ensure that the original agronomic qualities of the parent line are retained. Furthermore, naturally-occurring resistance genes are typically only

effective against specific genotypes of a single fungal or bacterial species. However, DRR206 is known to be activated in pea in response to a variety of different species of bacteria and fungi, suggesting that DRR206 will confer resistance to a broader range of pathogens. This is supported by the fact that DRR206 confers resistance to other pathogens, for example, *Sclerotinia sclerotiorum* and *Rhizoctonia solani*. Furthermore, as the DRR206 sequence is not naturally-occurring in *B. napus*, the presence of the gene in progeny from a cross can be verified using molecular methods rather than infection phenotype. Thus, blackleg resistance can be quickly and easily transferred to any cultivars of *B. napus*.

The invention will now be described by way of examples, although it is to be understood that the invention is not limited to these examples.

EXAMPLE I – TRANSGENIC *B. NAPUS* PLANTS

The 35S-constructs were prepared as follows: genomic or cDNA coding sequences for pea PR10 (cDNA) (Drr49) (Culley et al, 1995a, *Plant Physiol* **109**: 772), chitinase (genomic) (Chang et al, 1995, *Plant Mol Biol* **28**:105-111), DRR206 (genomic) (Culley et al, 1995b, *Plant Physiol* **107**: 301-302) or defensin Drr230 (cDNA) (Chiang and Hadwiger, 1991, *Mol Plant-Microbe Interact* **4**:324-331) were cloned into T-DNA-based binary vector pBI121 (Clonetech Inc), replacing the GUS gene, as shown in Figure 1. The four 35S-defense genes, PR10.1, DRR206 (Fristensky et al, 1985), chitinase (Chang et al, 1995) and defensin (Chiang and Hadwiger, 1991) were introduced into *B. napus*, *Bassica napus cv.*, Westar using *Agrobacterium tumefaciens* strain MP90 by cotyledonary petiole transformation (Moloney et al, 1989, *Plant Cell Reports* **8**:238-242). Seeds from Westar and from transformed plants were grown in Metro-mix™ in a growth chamber with a day/night regime of 22/15^{O}C and 16/8 photoperiod.

EXAMPLE II – INOCULUM

Pycnidiospores of *L. maculans*, pathogenicity group 2 (PG2) from isolate 86-12, (Dr. R. Rimmer, University of Manitoba) were initially obtained from infected cotyledons, according to the modified method from Williams (Williams, 1985, Crucifer Genetics Cooperative Resource Book, University of Wisconsin: Madison, Wisconsin). Diseased tissue was surface-sterilized in 2% sodium

hypochlorite for 5 minutes, and then placed on V-8 juice agar medium containing 0.75% $CaCO_3$, 0.1% streptomycin sulphate and 0.04% rose bengal. The plates were incubated under constant light at 22-24^{o}C for one week until sporulation. Strips of the *L. maculans* culture were placed on the prepared V-8 medium and incubated in the same conditions for another week. The spore suspension was then collected by scraping the surface of a plate with a sterile glass slide to release pycnidiospores in 6 ml of sterile distilled water. This suspension was then filtered through sterile miracloth and centrifuged. The spore suspension was stored in a –80^{o}C freezer. The spore concentration was adjusted to $2x10^{7}$ spores/ml for inoculation.

EXAMPLE III – PLANT INOCULATION

Cotyledon inoculation Cotyledons of eight-day plants were wounded with a syringe needle and 10 µl of the pycnidiospore suspension was dropped onto the lesion. Disease scores were evaluated at 10 days post-inoculation using a disease rating scale based on lesion size, tissue blacking, tissue collapse and presence of pycnidia (Williams and Dehwiche, 1980, Eucarpia "Cruciferae 1979" conference, Wageningen : pp164-170). Cotyledons from Westar control and from transgenic plants with the DRR206 and defensin genes were infiltrated with the spore suspension and the samples were collected post inoculation each day for 8 days. Tissue was fixed in glacial acetic acid and absolute ethanol (1:2, v/v). The fixed solution was changed twice daily for three days. Unstained tissue was then observed under the microscope to observe the morphological characteristics of the blackleg fungus, as described below.

Adult plant inoculation The lower portion of the stem (close to the third node), was inoculated at the 5-6 leaf stage. 10 µl of pycnidiospore suspension was injected into the stem and the plants were kept in a humid environment for 24 hours. The disease rating scale is based on length, colour of lesions and presence of pycnidia (0-5, 0: No infection; 1: L<10 mm, W<25%, 2: L=10-19 mm, W=25-50%; 3: L=20-29 mm, W=51-75%; 4: L≥30 mm, W≥76-100%; 5: plant dead. L = lesion length; W = lesion area as a fraction of stem cross-section) (Cargeeg et al, 1980, *Aust J Aric Res* **31**:37-46, incorporated herein by reference).

Bioassays for antifungal activity Uninoculated young leaves of *B.*

napus cv. Westar or transgenic lines carrying DRR206 or defensin were ground under liquid N_2 and 1 ml of sterile distilled water was added per gram of fresh weight. Insoluble components were removed by centrifugation at 4000 g. The extract was filtered through a 0.2 μM filter. *L. maculans* pycnidiospores were added to the extract to a concentration of 1 x 10^6 spores/ml and incubated on a shaker at 120 rpm at room temperature for 72 hours. Germination was quantified in a hemocytometer, using four duplicate grids per treatment. Three hundred pycnidiospores were counted in each grid. After 5 days incubation, cultures were centrifuged at 4000 g, pellets were dried in centrifuge tubes in a 65°C oven for two days and weighed to measure dry weight of hyphae.

EXAMPLE IV – ESTIMATION OF TRANSGENE COPY NUMBER

DNA extraction procedure was the same as described for PCR (Dellaporta et al, 1983, *Plant Mol Biol Rep* **1**:19-21, incorporated herein by reference) with an additional two-time phenol (pH 8.0) extraction and RNase treatment for 30 minutes. Genomic DNA was restricted with HindIII and SstI (PR10.1) or HindIII and EcoRI (chitinase, defensin and DRR206). The UV crosslinked Zeta-probe blotting membrane was hybridized with ^{32}P-labeled random primed probe in 0.5 M Na_2HPO_4, pH 7.2 and 7% SDS at 65°C. A 0.5 kb fragment containing the NptII gene from pBI121 was used as a probe. Filters were washed twice with 40 mM Na_2HPO_4, pH 7.2 and 5% SDS at 65°C for 30 minutes, and twice with 40 mM Na_2HPO_4, pH 7.2 and 1% SDS at 65°C for 30 minutes according to the manual from Bio-Rad laboratories.

EXAMPLE V – IDENTIFICATION OF TRANSGENIC PLANTS BY PCR

DNA extraction was performed according to a modified protocol from Dellaporta, described above. Leaves (0.5 g) were added to 500 μl of extraction buffer (100 mM Tris-HCl (pH 8.0), 50 mM EDTA, 500 mM NaCl, 1.25% SDS) and incubated for 20 minutes at 65°C. Next, KOAc was added to a final concentration of 1M. The solution was kept on ice for 20 minutes and then extracted with chloroform:isoamyl alcohol (24:1). For the PCR reactions, mixture contained 1.5 mM MgCl, 0.2 mM dNTP, 0.5 μM of the specific primer mix and 2.5 units of Taq DNA polymerase. Gene-specific primers were as follows: for DRR206: oC206+1 (aattccaaacaagagaaagcc) and oC206-2 (cttgatataaacaccaagtcg); for defensin:

oS39b+3 (caagaaatagtggtgagtgaa) and oS39b-4 (gcgacaaccacgtgattttg). PCR cycles as follows: first cycle: 94°C for 3 minutes, 55°C for 45 seconds, 72°C for 1 minute; 2-29 cycles: 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 1 minute.

EXAMPLE VI – RNA EXTRACTION AND NORTHERN BLOTTING

RNA was extracted using a procedure modified from Logemann et al, 1987, *Analytical Biochemistry* **163**:16-20, incorporated herein by reference. Leaves (0.5 g) were added into the tube containing 750 µl of extraction buffer (4 M Guanidine isothiocyanate, 0.1 M Tris-HCl, pH 7.5, 10 mM EDTA, 1% mercaptoethanol), mixed well and centrifuged for 20 minutes at 4°C. The supernatant was extracted using phenol and chloroform:isopropyl alcohol (24:1) three times. RNA was precipitated with 0.7 volumes of cold 95% ethanol and 0.2 volumes 1 M acetic acid at –70°C for 2 hours, then washed with 3 M NaOAc (pH 4.8) and 70% ethanol. Dried RNA was resuspended in DEPC-treated sterile distilled water. The procedure of hybridization and washing was the same as for southern blotting, described above.

EXAMPLE VII – PROTEIN EXTRACTION AND WESTERN BLOTTING

Three grams of leaves were ground in liquid N_2 and extracted with 6 ml extraction buffer (50 mM Tris-HCl (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 2mM thiourea, and 1.5% (w/v) polyvinylpyrrolidone) for 2 hours at 4°C under continuous stirring. Following centrifugation, $(NH_4)_2SO_4$ was added into the supernatant to a 75% relative saturated concentration. Proteins were precipitated overnight, then dialyzed against 5 mM Tris-HCl buffer, and lyophilized (Terras et al, 1992, *J Biol Chem* **267**:15301-15309). The crude proteins from DRR206 transformants, from untransformed Westar and from pea pods at 48 h.p.i. were run through Tricine-SDS-polyacrylamide gels. The separating gel contained 12% acrylamide and Biorad low MW marker™ (66.0 kD – 14.2 kD). One gel was stained in 0.25% Coomassie Brilliant Blue R 250 in 45% methanol and 10% acetic acid and destained in 7.5% acetic acid and 10% methanol (Schagger and Jagow, 1987, *Analytical Biochemistry* **166**:368-379). A duplicate gel was electroblotted onto nitrocellulose membrane and detected by the Immun-Blot colorimetric assay kit™ (GAR-AP, Bio-Rad).

EXAMPLE VIII – TRANSFORMATION OF CONSTITUTIVELY-EXPRESSED CONSTRUCTS INTO *B. NAPUS* AND SELECTION OF SINGLE COPY LINES

Genomic or cDNA coding sequences for pea PR10 (Drr49) (Culley et al, 1995a), chitinase (Chang et al, 1995), DRR206 (Culley et al, 1995b) or defensin Drr230 (Chiang and Hadwiger, 1991) were cloned into T-DNA-based binary vector pBI121, replacing the GUS gene, as shown in Figure 1 and as discussed above. Constructs were directly transfected into *A. tumefaciens*, and cotyledonary petioles were transformed by cocultivation, as described above.

Because unlinked loci assort independently, genetic analysis of traits governed by more than one locus is very complex. Also, previous studies have shown that multiple T-DNA insertions can lead to gene silencing (Flavell, 1994, *Proc Natl Acad Sci* **91**:3490-3496; Taylor, *The Plant Cell* **9**:1245-1249). For these reasons, T_O plants (i.e. – initial transformants) were screened by DNA gel blot hybridization to identify transformants bearing single T-DNA insertions. Transgene copy numbers ranged from 1 to 11 as estimated by southern blot analysis, as shown in Figure 2. The ratio of plants containing single copy inserts to the total number of transgenic lines were: PR10.1: 7/28; chitinase: 3/22; DRR206 : 7/28; defensin: 2/24. All further work was carried out in single copy lines.

EXAMPLE IX – DISEASE RESISTANCE TO *L. MACULANS* ISOLATE PG2 IS CORRELATED WITH STRONG EXPRESSION OF PEA DRR206 AND DEFENSIN

For the first round of screening of single copy lines, leaf tissue from 5 T1 plants was pooled and expression of transgenes assayed by RNA gel blot analysis, as described above. In parallel experiments, cotyledons of 20 8-day seedlings were grown for each line, and inoculated with compatible blackleg PG2. After scoring at 10 d.p.i., cotyledons were removed to prevent colonization of stems. For the cotyledon assay, plants showing no disease symptoms at all were excluded from scoring to control for the possibility of escapes. Also, two of four lobes were inoculated per cotyledon, and the lobe showing the highest disease score was used for scoring. Cotyledon assay scores must therefore be considered conservatively high. At the 5-6 leaf stage, stems of the same plants were inoculated with *L. maculans*, and symptoms scored 5 weeks after inoculation. The

results of these three experiments are compared in Table 1 and Figure 3.

Transgenic lines containing PR10.1 exhibited similar disease scores to the Westar control, and mRNA levels for this gene ranged from high to undetectable. The transformed lines without mRNA accumulation were used as controls for disease scoring, in addition to the untransformed, as shown in Table 1 and Figure 3. Among three lines containing chitinase constructs, only GN2-2#13 and GN2-2#31 expressed chitinase mRNA. These lines showed only a small decrease in adult plant disease. The strongest effect was seen with DRR206. Five lines exhibiting DRR206 expression also showed significantly lower disease scores, compared to lines with no detectable expression. T2 plants from GN3-4#22 also showed lower disease scores in the cotyledon assay. Of two lines carrying pea defensin, the one line that exhibited mRNA expression for this gene also had slightly lower disease scores in adult and cotyledon assays with both T1 and T2 plants.

The effect of the pea DRR206 and defensin on blackleg resistance in transgenic *Brassica* are shown in Figure 4. On untransformed Westar cotyledons, brown lesions with black pycnidia are evident, as shown in Figure 4a. In T2 plants bearing the defensin gene, browning is limited to a smaller region near the inoculation site, and no pycnidia are in evidence, as shown in Figure 4b. In T2 DRR206 transgenic plants, no browning, outside of the site wounded during inoculation, are seen, as shown in Figure 4c. Adult plant resistant phenotypes for T1 are shown in Figure 4d. Stem-inoculated untransformed Westar plants die after blackleg inoculation, exhibiting almost complete girdling of the stem, as shown in Figures 4d and 4e. Stem lesions are reduced in defensin transformants, and plants remain viable, as shown in Figures 4d and 4f. It is of note that stem lesions are not evident on DRR206 transformants, as shown in Figure 4g.

EXAMPLE X – VERIFICATION OF TRANSGENE EFFECTS IN T2 PLANTS

Resistance co-segregates with DRR206 The T1 lines with lowest disease scores, GN3-4#22 (DRR206) and GN4-2#15 (defensin) were self-propagated. T2 plants containing the genes were identified by PCR analysis using primers specific for pea DRR206 and defensin. T2 progeny from a single parent segregated 3:1 (presence:absence of the gene) based on PCR results, as shown

in Table 2. T2 plants from the GN3-4#22 line with the DRR206 gene uniformly exhibited significantly improved resistance to *L. maculans* when compared to T1 plants and to the Westar control. Their cotyledon disease scores were only 2.6 ± 0.5 and adult disease scores were 1.4 ± 0.6, as shown in Table 2. In contrast, T2 plants from the GN4-2#15 line with defensin did not show significantly more resistance than T1 progenitors. The disease scores of cotyledon and adult plants were 7.2 ± 0.5 and 3.4 ± 0.9 respectively, as shown in Table 2.

Resistance to *L. maculans* in DRR206 lines is associated with decreased spore germination Cotyledon inoculation sites of resistant T2 plants bearing defensin or DRR206 genes were compared in light microscopy, with inoculation sites in untransformed Westar plants. As shown in Figures 4h and 4l, Westar and defensin transgenics both exhibit substantial intercellular hyphal growth at the site of inoculation. DRR206 transgenic plants, on the other hand, show a marked reduction in the density of hyphae visible in tissue, as shown in Figure 4j.

To further investigate the inhibition of fungal growth, extracts of water-soluble components from leaves of Westar or T2 plants transgenic for either defensin or DRR206 were inoculated with *L. maculans* PG2. After incubation for 72 hours, extracts from either defensin or DRR206 transgenic plants showed significant decreases in germination, compared to extracts from untransformed Westar plants, as shown in Table 3. A reduction of recoverable dry weight of hyphae at five days post-inoculation was observed only when DRR206 extracts were used, as shown in Table 3.

Infection phenotypes of Westar with defensin and DRR206 transgenic plants were also compared. In contrast to the usual cotyledon assay, in which a small site is inoculated using a needle, a large area of the cotyledon was infiltrated with PG2 pycnidiospores using a syringe with no needle. As shown in Figure 4k, untransformed Westar plants exhibit browning, indicative of necrosis, at 8 days post-inoculation. Defensin transgenics also exhibit necrosis. DRR206 transgenics exhibit chlorosis or yellowing of inoculated tissue, indicative of a hypersensitive response.

DRR206 protein expression The expression of the DRR206 protein

was verified by protein gel blots, as described above and as shown in Figure 5, using polyclonal antibodies from rabbits injected with DRR206 expressed in *E. coli* (Culley et al, 1995, *Plant Physiol* **107**:301-302). As a positive control, proteins were extracted from pea pods 48 hours after inoculation with *F. solani* f. sp. *phaseoli*. Under these conditions, pea expresses a basic resistance to *F. solani* f. sp. *phaseoli*, along with a strong induction of DRR206 mRNA (Fristensky et al, 1985). In pea tissue, two major bands at apparent molecular weight classes 25 and 22 kD, along with a minor band at 28 kD, were detected. The molecular weight of the DRR206 polypeptide, inferred from the complete sequence of the genomic clone, is 20.4 kD, while the molecular weight of the purified peptide was previously shown to be 23 kD (Culley et al, 1995b). The 28 kD band was also faintly visible in proteins from untransformed Westar and is therefore presumably an unrelated protein that also cross-reacts with the antisera. Neither the 25 kD nor 22 kD proteins were visible in Westar-derived protein extracts. Proteins from T1 and T2 resistant DRR206 transgenic plants exhibited bands which co-migrated with the 25 kD and 22 kD bands from pea. The presence of two bands in pea and transgenic *Brassica* suggests that DRR206 undergoes post-translational modifications.

EXAMPLE XI - T3 AND T5 TRANSGENIC PLANTS EXPRESSING DRR206 ARE ALSO RESISTANT TO AGGRESSIVE *L. MACULANS* ISOLATES PG3 AND PG4.

To determine whether resistance to blackleg in DRR206 transgenic plants was limited to a single genotype of the pathogen or was broader in scope, adult plant assays, as described above, were carried out using *L. maculans* isolate PG3 (Rimmer and van den Berg, 1992). As shown in Figure 6, untransformed control plants (cv. Westar) had an average disease score of 5, while T3 plants derived from transformant GN3-4#22 all showed substantially lower disease scores in adult plants, as shown in Table 4. Similarly, T5 plants also showed lower disease scores when inoculated with PG4 (Rimmer and van den Berg, 1992), as shown in Table 5.

EXAMPLE XII - T3 TRANSGENIC PLANTS EXPRESSING DRR206 EXHIBIT SMALLER LESIONS WHEN INOCULATED WITH *SCLEROTINIA SCLEROTIORUM*.

S. sclerotiorum is a highly-agressive fungus with one of the widest host ranges of any known pathogen. A recent survey of the literature described 408 species from 278 genera and 75 families that were susceptible to *Sclerotinia* (Boland and Hall, 1994, *Can. J. Plant Pathol.* 16:93-108). In most crops, few sources of genetically determined resistance have been found. Consequently, genes conferring even partial resistance to *Sclerotinia* could be useful for genetic engineering in a broad range of crop species.

Sclerotia from *S. sclerotiorum* clone 38 (from Dr. L.M. Kohn, Dept. of Botany, Univ. of Toronto, Toronto, Canada) were incubated on potato dextrose agar (PDA) for 3 days at room temperature. Mycelial plugs (3 mm) were cut and transferred to new PDA plates and incubated at 24°C, 48 hr. Fresh plugs containing new mycelia from the growing margin of each colony were cut and placed in the middle of the first two leaves of each plant. Plants were incubated in a mist chamber for 2 days. The misting chamber was located in a growth room and illuminated with ambient light. The diameters of necrotic lesions were measured after 48 hr.

Figure 7 shows the diameter of lesions on *B. napus* plants inoculated with *Sclerotinia*. On untransformed cultivar Westar, the mean lesion size is 2.2 cm (mean of 10 plants inoculated on 4 leaves). In Westar-derived T3 lines transformed with DRR206, line GN3-4#22-19 showed a mean lesion size of 1.7 cm, and GN3-4#22-2 a mean lesion size of 1.5 cm (mean of 20 plants inoculated on 4 leaves). While resistance was not complete, these results suggest that DRR206 may act to decrease the rate of colonization of tissue by *Sclerotinia*. Data is also shown in Table 7.

EXAMPLE XIII – SURVIVAL OF DRR206 TRANSFORMED LINES FOLLOWING *Rhizoctonia solani* INFECTION

To determine whether resistance in DRR206 transgenic plants extended to other pathogens, plants were inoculated after 8 day planting and disease scores were tallied at 8 days postinoculation. "Plant death" was typically obvious, with lodging and complete wilting of all above-ground parts of the seedling, as shown in Table 6. As can be seen, transgenic lines were much more resistant to *Rhizoctonia solani* compared to the Westar control. The significance of

these observations is that DRR206-mediated resistance is clearly not specific for a single fungal isolate, but is effective against a range of fungal species with different modes of pathogenicity, as discussed below.

EXAMPLE XIV – DISCUSSION

For a single, constitutively-expressed foreign gene to confer disease resistance to a susceptible plant, there are three broad categories or possible functions:

(1) The gene product itself, or an enzymatic product produced by it, is directly active against the pathogen.

(2) The gene influences the expression of resistance in the host, either through direct (epistatic) effects on defense regulatory pathways, or as an indirect (pleiotropic) effect leading to the induction of defense pathways.

(3) The gene product provides some function that is compatible with the host's defenses, but is normally lacking in the incompatible interaction.

Neither pea PR10 nor basic chitinase appears to meet any of these criteria in the interaction of transgenic *B. napus* with *L. maculans*. When this same PR10 gene was transformed into potato (Chiang et al, 1993), tubers inoculated with *Verticillium dahlie* showed an almost 50% reduction in lesion size, compared to untransformed controls. In *verticillium*-infested soil, PR10 transgenic potatoes showed a decrease in wilting, accompanied by about a 2-fold increase in tuber yield, compared to untransformed controls. Since our experiments employ a different host/pathogen system, there may be several reasons why PR10 did not enhance resistance.

The pea chitinase (Pissa;Chia;1;2) used in this paper is a PR3 endochitinase in the same subfamily as the bean chitinase (Phavu;Chia;1;3) used previously (Broglie et al, 1991). Specifically, these proteins share 87% amino acid identity. Constitutive expression of the bean chitinase decreased seedling mortality in both transgenic tobacco and *B. napus* cv. Westar to *Rhizoctonia solani* (Broglie et al, 1991). In our work, the same cultivar of *B. napus* was used. The simplest explanation therefore is that *L. maculans* is less sensitive to suppression by PR3-type chitinases than *R. solani.*

While the strongest enhancement of resistance to blackleg was seen

in DRR206 transgenic lines, the function of this gene remains unknown. In pea, DRR206 is induced strongly by an incompatible race of *F. solani*, and more weakly by a compatible race (Fristensky et al, 1985). With compatible isolates of *P. syringae*, an early induction of DRR206 is seen, which subsides after 12 h.p.i. In gene-specific incompatible interactions with *P. syringae*, DRR206 is induced early and expression remains strong for at least 30 h.p.i (Daniels et al, 1987). In *F. oxysporum*-infested soil, DRR206 is very strongly expressed in resistant isoline Vantage, and only weakly induced in susceptible isoline M410 (Hadwiger et al, 1992).

Despite the strong differences in expression seen with DRR206 in resistant compared to susceptible interactions in pea, no homologues of this gene have yet been cloned in studies of other plant/pathogen interactions. Therefore, no clues as to its function can be found in other systems.

Our results do show that DRR206 transgenic plants produce water-soluble antifungal activities, as shown in Table 3. Decreased hyphal growth is also seen at infection sites, as shown in Figure 4j. Furthermore, resistance to blackleg can occur both in cotyledons (Figures 4c and 4k) and in adult stems (Figures 4d and 4g), suggesting that DRR206-mediated resistance is not limited to a single developmental stage or tissue. The almost complete lack of symptoms beyond the site of the pinprick inoculation in cotyledons indicates that DRR206 must act early in the infection process, for example, inhibiting spore germination or hyphal growth, rather than later, for example, inhibiting subsequent colonization. Most intriguing is the observation that DRR206 transgenic plants appear to express a hypersensitive response (HR) to fungal inoculation as evidenced by chlorosis and tissue collapse (Figure 4k), despite the fact that the 35S-DRR206 gene itself is not inducible. This suggests that constitutive expression of DRR206 in *B. napus* in some way potentiates the HR, perhaps through lowering some threshold mechanism that normally prevents an HR in a compatible interaction. Regardless of the precise mechanism of its action, the ability of DRR206 to confer blackleg resistance in transgenic *B. napus* substantiates a role for this gene in plant defense.

Pea defensin is less effective than DRR206 in conferring resistance

to *L. maculans.* Extracts from defensin-transgenic plants inhibit spore germination and growth. This is consistent with previous reports showing that radish defensin can cause hyper-branching and decreased growth of *Alternaria longipes* in-vitro (Terras et al, 1995). Inhibition of fungal growth or germination by extracts from defensin transgenic plants is less pronounced that that seen with DRR206, as shown in Table 3. This is consistent with the fact that DRR206 transgenics also showed lower disease scores, as shown in Tables 1 and 2 and Figure 3. In the cotyledon assay, defensin plants allowed infection to spread to a wider area than DRR206. One potentially important difference between defensin transgenics and the Westar control was the absence of pycnidia in lesions of defensin plants (Figures 4a and 4b). Defensin transgenics also exhibited smaller stem lesions compared to the Westar control (Figures 4e and 4f) but allowed a similar degree of hyphal growth (Figures 4h and 4l). Finally, defensin transgenics did not exhibit the kind of HR seen in DRR206 transgenics. Taken together, these comparisons suggest that the resistance mediated by DRR206 is qualitatively different from defensin-mediated resistance.

Furthermore, it has been shown that DRR206-mediated resistance is not specific for a single fungal isolate but is effective against a range of different fungi with different modes of pathogenicity. For example, blackleg fungus is a hemibiotroph whereas *Scleotinia* is a necrotroph. Similarly, blackleg infects above-ground portions of the plant whereas *Rhizoctonia* is a root pathogen.

In addition, it is important to remember that the DRR206 gene comes from pea, and is activated by both bacteria and fungi in pea. Consequently, there is no reason to believe that the effectiveness of DRR206 would be limited to *Brassica napus.* Clearly, support is shown for the effectiveness of DRR206-mediated resistance in many plant species, against many pathogens.

It is of note that other suitable promoters known in the art, such as, for example, inducible promoters or tissue specific promoters may be used in the construction of vectors for expression of plant defense genes, for example, DRR206 and defensin. Specifically, the inducible promoters may be responsive to pathogen attack. Examples of pathogen-inducible promoters include the pea DRR49a promoter (Culley, D. et al. (1995) *Plant Physiology* **109**:722), the pea

DRR206c promoter (Wang, Y. et al., (1999) *Molecular Plant-Microbe Interactions* **12**:410-418), the tobacco PR-1a promoter (Strompen, G. et al., (1998) *Plant Molecular Biology* **37**:871-883), or the bean chitinase promoter (Roby, D., et al. (1990) *Plant Cell* **2**:999-1007.). As will be apparent to one knowledgeable in the art, the list above is for illustrative purposes and is in no way limiting.

Since various modifications can be made in our invention as herein above described, and many apparently widely different embodiments of same made within the spirit and scope of the claims without department from such spirit and scope, it is intended that all matter contained in the accompanying specification shall be interpreted as illustrative only and not in a limiting sense.

Table 1. Specific RNA accumulation and disease scores in canola transformed lines with defense genes. All plants are T1 transformants, except where indicated as T2.

Lines	Gene	RNA*	Disease scores**	
			Cotyledon	Adult Plant
Westar	-	-	9.0±0.0 a	4.8±0.2 a
GN1-2#4	PR10.1	+	8.8±0.3 a	4.6±0.1 a
GN1-4#51-1	PR10.1	-	8.9±0.2 a	4.7±0.2 a
GN1-5#18	PR10.1	+	8.8±0.4 a	4.4±0.1 a
GN1-5#22	PR10.1	+	8.8±0.3 a	4.2±0.5 a
GN1-5#53-1	PR10.1	+	8.9±0.1 a	4.6±0.2 a
GN1-5#54-1	PR10.1	+	8.7±0.4 a	4.8±0.1 a
GN1-5#54-2	PR10.1	+	8.8±0.3 a	4.6±0.2 a
GN2-4#32-2	Chitinase	-	8.9±0.4 a	4.9±0.4 a
GN2-2#13	Chitinase	+	8.9±0.1 a	3.8±0.8 ab
GN2-2#31	Chitinase	+	8.6±0.2 a	3.6±0.9 ab
GN3-2#11-1	Drr206	-	8.9±0.4 a	4.5±0.3 a
GN3-2#11-2	Drr206	-	8.8±0.2 a	4.8±0.2 a
GN3-5#26	Drr206	+	8.8±0.1 a	3.4±1.1 b
GN3-5#23	Drr206	+	8.6±0.1 a	3.0±1.2 bc
GN3-1#5	Drr206	+	7.8±0.5 b	2.9±1.3 bc
GN3-5#24	Drr206	+	8.4±0.2 a	2.6±1.3 c
GN3-4#22	Drr206	+	7.9±0.5 b	2.5±1.4 c
GN3-4#22(T2)	Drr206	+	2.6±0.5 c	1.4±0.6 d
GN4-1#12	Defensin	-	9.0±0.1 a	4.7±0.1 a
GN4-2#15	Defensin	+	7.9±0.1 b	3.5±1.0 b
GN4-2#15(T2)	Defensin	+	7.2±0.5 b	3.4±0.9 b

*+ : mRNA expressed; - : no detectible mRNA expression;

** means±SE (standard error of the mean) of 20 plants; lines with the same letter are not significantly different at p=0.05. (Duncan's Multiple Range Test)

Table 2. Disease score co-segregates with defense T-DNA insertions in T2 plants:

	Drr206		Defensin	
PCR band	+	-	+	-
Cotyledon score	2.6±0.5	8.6±0.5	7.2±0.5	8.5±0.6
Adult score	1.4±0.6	4.5±0.2	3.4±0.9	4.6±0.2
Observed ratio	25	8	24	8
Expected ratio	3	1	3	1
Probability[a]	0.99>p>0.95		p>0.99	

+: presence of gene; -: absence of the gene
a: a fit to the expected ratio is accepted if p>0.05 (chi-square test)

Table 3. Germination rate of pycnidiospores of *L. maculans* and dry weight of hyphae of *L. maculans*.

	Westar	Defensin	Drr206
Germination rate %	30.9±1.2	15.6±1.5	5.8±1.0
Dry weight mg per ml of culture	1.6±0.2	1.3±0.4	0.8±0.2

Table 4. T3 transg nic lines inoculated with *Leptosphaeria maculans* PG3

Lines	Adult plant stem inoculation		
	AVG		STD
Westar	5.00	a	0.00
GN 3-4 #22-35	2.50	b	0.50
GN 3-4 #22-19	1.67	c	0.68
GN 3-4 #22-2	0.80	c	0.47

Each Westar has 15 plants and each transformed line has 20 to 28 plants. Two replicates were done for each line. Scores with the same letter are not significantly different at p=0.05. (Duncan's Multiple Range Test).

Table 5. T5 transgenic lines inoculated with *Leptosphaeria maculans* PG4

Lines	Cotyledon			Adult		
	AVG		STD	AVG		STD
Westar	9.00	a	0.00	5.00	a	0.00
22-19-5-3	1.78	b	0.84	0.80	b	0.40
22-19-5-20	2.48	b	0.94	0.56	b	0.49
22-19-4-20	2.84	b	1.01	1.09	b	0.51
22-2-1-17	2.45	b	0.93	0.86	b	0.34
22-19-5-7	3.83	b	1.05	1.10	b	0.30
22-19-4-21	2.87	b	0.97	0.85	b	0.35
22-14-10-12	1.50	b	0.87	1.00	b	0.40

Table 6. Disease Evaluation of DRR206 Transformed Lin s inoculated with *Rhizoctonia solani*

T6 transgenic lines inoculated with *Rhizoctonia solani* RC6

Lines	Plant death (%)
Westar	94 a
GN3-4#22-1-19-5-3-12	32 b
GN3-4#22-1-19-5-3-6	21 c
Quinta	16 c
GN3-4#22-1-19-5-3-17	5 d

Plants were inoculated after 8 day planting and disease scores were scored after 8 days postinoculation. "Plant death" was typically obvious, with lodging and complete wilting of all above-ground parts of the seedling. Scores with the same letter are not significantly different at p=0.05. (Duncan's Multiple Range Test).Westar or Quinta(2 replicates for a total of 30 plants for each) and each transformed line (2 replicates for a total of 46 to 54 plants) were done.

Table 7. Disease Evaluation of DRR206 Transformed Lines inoculated with *Sclerotinia sclerotiorum*

Each Westar has 10 plants and each transformed line had 10 to 20 plants. Four replicates were done for each line. Scores with the same letter are not significantly different at p=0.05. (Duncan's Multiple Range Test).

T3 transformants

Lines	Mean lesion diam. (cm.)	SE
Westar	2.26 a	0.57
Gn3-4#22-19	1.73 b	0.53
GN3-4#22-2	1.57 b	0.53

T4 transformants

Lines	Mean lesion diam. (cm.)		SE
Westar	3.22	a	0.14
22-19-3	2.13	b	0.67
22-19-5	2.27	b	0.51

T4 transformants

Lines	Mean lesion diam. (cm.)		SE
Westar	2.65	a	0.24
22-2-4	1.93	b	0.59
22-2-7	1.23	b	0.74
22-2-10	1.44	b	0.64
22-2-36	1.48	b	0.61

T5 transformants

Lines	Mean lesion diam. (cm.)		SE
Westar	3.14	a	0.29
22-19-4-21	2.44	b	0.17
22-19-5-20	2.02	b	0.31
22-19-5-3	2.15	b	0.14
22-19-4-20	2.15	b	0.23
22-2-1-17	2.33	b	0.15